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HISTORICAL REVIEW OF RESEARCH ON PROTEIN KINASE C IN LEARNING AND MEMORY

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Contents

Abstract

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1. In 1977, the discovery of a new type of kinase was reported, which turned out to be a receptor for phorbol esters. Thereafter, several mechanisms regulating PKC activity and various PKC subtypes have been discovered.

2. A role for PKC in synaptic plasticity and information storage has been postulated in the mid-1980s. An important role for PKC has since been suggested in several learning and memory models, in which persistent changes in the activation of PKC outlasting the initial stimulating event are thought to be crucial.

3. A vast number of experiments have further substantiated a role of PKC in learning and memory using molecular genetic, behavioral, pharmacological, electrophysiological or immunocytochemical approaches in the late 1980s and the 1990s. PKC research of the past decade or so of has shown some exciting aspects of the putative role of PKC in synaptic plasticity and information storage.

4. The authors have provided highlights (Table 1) on research on PKC.

Keywords: discocery, history, isoforms, learning and memory, long-term potentiation, models, phorbol ester receptor, Protein kinase C (PKC), synaptic plasticity, translocation.

Abbreviations: diacylglycerol (DAG), intermediate and medial hyperstriatum ventrale (IMHV), long-term potentiation (LTP), phorbol-12,13-dibutyrate (PDBu), phosphatidylinositol (PI), phospholipase A_2 (PLA_2), protein kinase C (PKC), Receptors for Activated C Kinase (RACKs)

1. Introduction

The identification of the phenomenon of protein phosphorylation, first described in the early 1930s, together with the realization of the importance of such protein modifications back in the early 1950s have been two major breakthroughs in our understanding in the regulation of key cellular processes. Protein kinases phosphorylate many cellular proteins, catalyzing the transfer of phosphate to certain aminoacid residues within proteins. Phosphorylation can alter the folding of the protein, and hence their function. In the late 1960s, cAMP-dependent protein kinase was discovered (Walsh et al., 1968), followed by cGMP-dependent protein kinase in 1970 (Kuo and Greengard, 1970). The discovery of a

novel, cyclic nucleotide-independent, protein kinase took place relatively recently in the late 1970s. The discovery of this enzyme and its impact on learning and memory research will be the main focus of this historical review.

2. Discovery of Protein Kinase C (PKC)

2.1 The Discovery of a Novel Protein Kinase

In 1977, co-workers from the Nishizuka group at Kobe University in Japan first reported to have found a new type of kinase in rat liver (Takai et al., 1977a), bovine cerebellum (Takai et al., 1977b) and in the cytosol of rat brain (Inoue et al., 1977), but it took another 2 years before it was referred to as PKC. They described the purification of a proenzyme from the soluble fraction of tissue homogenates. This proenzyme was initially thought to be activated by limited proteolysis with Ca^{2+}-dependent neural thiol protease (Takai et al., 1977a,b; Inoue et al., 1977). At that time they named the enzyme protein kinase M, probably since maximum enzyme activities were established in the presence of mM concentrations of Mg^{2+}. In fact, it turned out that they had isolated the catalytic portion of PKC, believing it to be a new type of unregulated protein kinase. The protease calpain fragmented PKC during the extraction of the kinase from tissue for purification. These catalytic fragments, which require Mg^{2+} but no second messengers to be active, are still called PKM. However, at that point in time neither the proenzyme nor its catalytic fragment had an obvious role in signal transduction pathways.

It was demonstrated by the same group that the precursor enzyme itself could be enzymatically fully active in the presence of Ca^{2+} and a 'membrane-associated factor' that was identified as a phospholipid later clarified to be phosphatidylserine (Kaibuchi et al., 1981; Orr et al., 1992). The protein kinase is activated in a reversible manner by attachment to membrane phospholipid in the presence of Ca^{2+} (Takai et al., 1979a,b). The apparently Ca^{2+}-dependent protein kinase precursor now received its final name: protein kinase C. Further analysis showed that a small amount of diacylglycerol (DAG; a minor component of the cellular lipids) significantly increases the affinity of this enzyme for Ca^{2+} and phospholipid (Takai et al., 1979; Kishimoto et al., 1980). DAG (and inositoltrisphosphate; Berridge, 1984) is produced by the metabolism of phosphatidylinositol bisphosphate (PI-turnover; the hydrolysis of phosphatidylinositol (PI) derivates by hormone or neurotransmitter-stimulated phospholipase C followed by resynthesis of PI). Interestingly, DAG permitted activation of PKC at resting intracellular Ca^{2+} levels (Kishimoto et al., 1980). PKC is usually present in an inactive form in the cytosol. Due to

the specific binding of PKC by DAG, which is transiently formed in the membrane, activation of PKC is accompanied by its translocation from the cytosol to the membrane (Kawahara et al., 1980). The duration and magnitude of the DAG signal determines the activation of PKC at the cellular membrane. Taken together, the *in vitro* results on PKC made it seem plausible that under physiological conditions PKC is activated when cells are stimulated by a wide variety of biological substances. It was therefore suggested that the release of DAG through the signal-induced breakdown of PI may trigger the activation of PKC *in vivo* (Kaibuchi et al., 1982; Nishizuka, 1984).

2.2 A Receptor for Phorbol Esters

Around 1982 it became clear that PKC was the receptor for tumor-promoting phorbol esters. Phorbol esters, which dissolve in the cell membrane, compete with DAG for the same binding site and activate PKC in a similar fashion (Castagna et al., 1982), thereby increasing the enzyme's affinity for Ca^{2+} approximately 10-fold. Since the observation that phorbol esters, like DAG, cause PKC translocation to membranes (Kraft et al., 1982; Kraft and Anderson, 1983), this and the accompanying activation of PKC has been the subject of extensive investigations. However, there was no proof for PKC being the sole target of phorbol esters (Nishizuka, 1986). In 1984, Moon et al. found effects of phorbol esters in membrane preparations from cells that lacked PKC, and in the mid-1980s the first discrepancies were found between physiological actions of phorbol esters and activation of PKC (Kreutter et al., 1985). It became clear that phorbol esters may sometimes be unsuitable for studies of the physiological activation of PKC; DAG is present only transiently in membranes, while phorbol esters may extend a usually limited phase of cellular response, thereby distorting the normal sequence of events (Nishizuka, 1986). Nowadays we know that there are also other receptors than PKC for phorbol esters (the 34kD *n*-chimaerin, for example (Hall et al., 1990)), calling into question some of the results of experiments obtained with phorbol ester to study the cellular function of PKC (Wilkinson and Hallam, 1994).

PKC gained much interest after the mid-1980s when the two major findings, i.e. that it was the main target of PI-turnover as well as the phorbol ester receptor, became widely known. Since the first reports in the late 1970s on PKC, a still increasingly growing body of papers appears in the literature each year (Fig. 1). Research on PKC has spread over many fields of biology and medicine, probably because of its diverse functions in signal transduction. Nowadays, approximately 40-50 papers are published on PKC every week, and the total number of PKC-related reports presently exceeds 17,000.

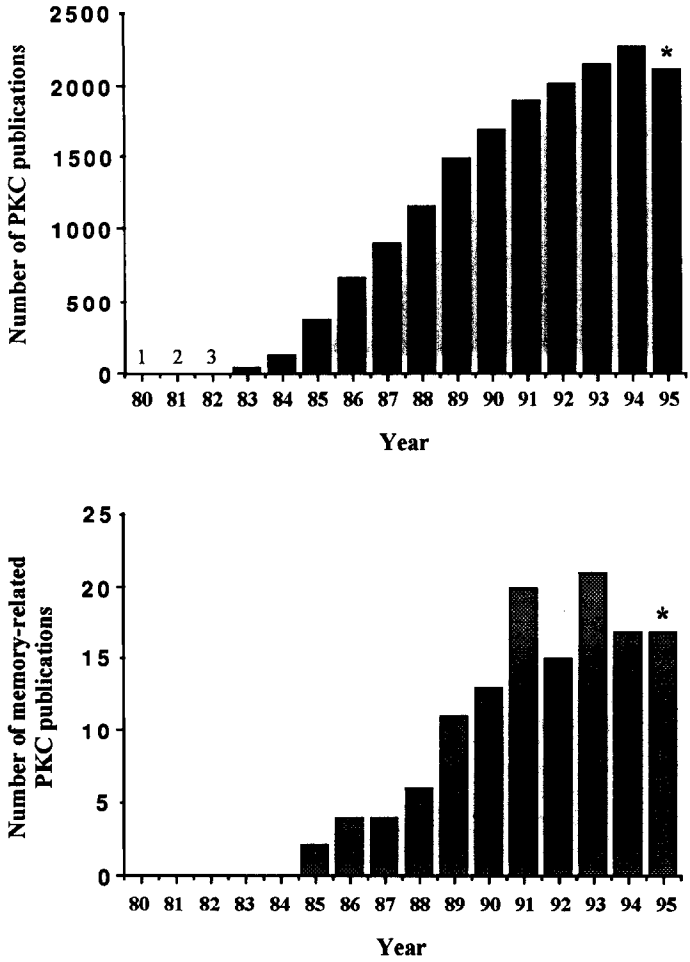

Fig. 1. Some statistics on publications related to PKC. After the initial reports of PKC in 1977 (Takai et al., 1977b; Inoue et al., 1977) it took several years (until approximately 1983) before considerable numbers of PKC-related papers appeared. After 1983, the number of yearly published reports on PKC increased steadily, with a top of 2290 in 1994, although probably even more papers appeared in 1995 (asterisks; the consulted database (Medline) did not yet include the last months of 1995).

2.3 The Discovery of Additional Mechanisms Regulating PKC Activity

Mechanisms for the regulation of PKC activity other than by DAG or phorbol esters were soon detected. For example, in the early 1980s there were already indications that PKC undergoes autophosphorylation (Kikkawa et al., 1982), and the mechanism of PKC autophosphorylation and the resulting change in the property of the kinase were determined in the mid-1980s (Huang et al., 1986a,b). Autophosphorylation increases the affinity of PKC for DAG and may therefore be important in the regulation of the activity subsequent to signal transduction. Experiments by Borner and co-workers (1989) provided the first evidence that PKC is phosphorylated *in vivo*. It is now known that PKC can be phosphorylated at several sites, each probably with its own functional consequence (Newton, 1995).

The first endogenous inhibitor putatively modulating PKC activity was isolated in the mid-1980s (Walsh et al., 1984; McDonald and Walsh, 1985). Nowadays several endogenous inhibitors are known, although it remains uncertain to what extent these substances function as a control mechanism regulating PKC activity *in vivo* (Melner, 1996). Also in the mid-1980s, it was first demonstrated that several cis unsaturated fatty acids, produced from phospholipids by the action of phospholipase A_2, were able to activate PKC providing a second pathway for PKC activation (McPhail et al., 1984; Murakami and Routtenberg, 1985). After these initial studies, many other cellular factors were found to modulate PKC activity, and the biochemical mechanisms underlying PKC activity have been shown to be rather complex (Nishizuka, 1995; Liu, 1996). These modulations contribute to the fine-tuning of PKC activity necessary to ensure regulated PKC activity in the midst of complex intracellular signaling pathways such as known to occur, for example, in learning and memory processes.

2.4 The Discovery of Various PKC Subtypes

Until the mid-1980s, PKC was thought to be a single entity, and it was known that the single polypeptide chain of about 77-80 kDa could be proteolyzed into two fragments of approximately 32 kDa (the regulatory domain with the DAG binding site) and the 51 kDa (the catalytic domain with the kinase activity) (Inoue et al., 1977). However, the existence of "proenzyme I and II" was recognized in the early 1980s (Kikkawa et al., 1982), although the exact nature of this apparent heterogeneity was not clarified. Three kinase activity peaks using chromatographic separation were first described by Huang et al. (1986b), which were designated as PKC I, II, and III. A new family of PKC-related genes (α, β, and γ) was identified in bovine, human, and rat genomes in 1986 (Coussens et al., 1986) and in rabbit genome

in 1987 (Ohno et al., 1987). Three members of the gene family were characterized and mapped to different chromosomal locations (Coussens et al., 1986; Knopf et al., 1986). It became soon certain that PKC I, II, and III are derived form γ, β, and α genes, respectively. Southern hybridization analysis (Coussens et al., 1986) suggested that an even larger number of PKC genes could exist, which turned out to be true in later years. Until the mid-1980s, Western blot analysis has shown a single polypeptide supporting the belief that PKC was a single protein. Using a different PKC purification procedure, Woodgett and Hunter (1987) were the first to describe a doublet of PKC in Western blots, providing evidence that they represented two distinct forms of PKC. PKC was thereafter proven to be a complex family of closely related structures (Pears, 1995; Nishizuka, 1995), and to date at least 12 PKC isozymes have been identified and classified into three groups based on their structure and cofactor regulation. The discovery of the diverse PKC isoforms contributed to our understanding of the mystery how a great diversity of messages in cellular communication can be generated by only a limited number of different components: apparently this was not only achieved by multiple receptors, but by multiple PKC isoforms as well. It has been demonstrated that PKC subtypes have an isoform-specific cellular localization in various tissues and cell types (Tanaka and Saito, 1992). Apparently, each isozyme of the PKC family has its own spatial organization and is present in the right intracellular compartment of the cell, presumably in association with its specific target substrate proteins (for further details, see Casabona and Ramakers et al., this issue).

3. Discovery of PKC as a Putative Key Protein in Synaptic Plasticity and Information Storage

It is believed that memories are stored via highly selective changes in the strength of synaptic connections between neurons in the brain. As such, storage of information in the brain appears to involve persistent, use-dependent alteration in the efficacy of synaptic transmission. Approximately a decade ago the first experimental data in support of a putative role of PKC in synaptic plasticity and information storage were reported. The chain of events leading to our current knowledge of PKC (Table 1) and its contribution to learning and memory will be outlined below.

3.1. The Link Between Receptor Stimulation and PKC Activation

Hokin and Hokin (1953; 1954) first demonstrated that acetylcholine induced a greatly increased rate of uptake of ^{32}P into phospholipids in pancreatic exocrine tissue. Acetylcholine, acting through

Table 1

Approximate Time Table of Relevant Mile Stones in Research on PKC.

Year	Event
1977	Discovery of protein kinase C
1978	
1979	Ca2+ and phospholipid dependent character of PKC
1980	Diacylglycerol stimulates PKC Translocation of PKC from cytosol to membrane increases the activity of PKC A role for PKC in signal transduction implicated
1981	
1982	PKC is a receptor for phorbol esters
1983	The role of PKC in signal transduction is firmly established
1984	Discovery of endogenous PKC inhibitors
1985	Immunocytochemical localization of PKC in brain tissue Modulation of membrane functions by PKC: a role for PKC in synaptic plasticity and information storage?
1986	[3H] phorbol esters used to localize PKC in brain tissue A role for PKC in LTP First evidence for PKC isoforms (conventional PKCs, α, β, and γ) The mechanism of autophosphorylation of PKC determined
1987	Discovery of various other PKC isoforms
1988	
1989	
1990	The role of PKC in synaptic plasticity and information storage is widely accepted
1991	A role for PKC in LTD Identification of receptors for activated PKC (RACKs)
1992	Different role of PKC isoforms in information storage (notably the brain specific γ-isoform)
1993	PKCγ-mutant mice reveal modified LTP but only mild learning deficits
1994	
1995	
1996	More than 100 PKC substrates are known

muscarinic receptors, also provoked increases in phospholipid metabolism in cerebral cortex slices. Later, Hokin and Hokin showed that these changes were largely confined to the hydrolysis of PI (Hokin and Hokin, 1958). In the late 1960s and early 1970s it became apparent that rapid enhancement of PI-turnover occurred in a large variety of tissues and could be evoked by numerous (extracellular) stimuli (Lapetina and Michell, 1973). Nevertheless, the physiological significance of such PI-turnover remained unclear until the late 1970s, when Takai et al. (1979a) provided experimental evidence that the cholinergic cell response involved activation of PKC by means of the production of DAG. Interestingly this way a direct link was established between (muscarinic) receptor stimulation and activation of PKC in the central nervous system. This direct link between receptor activation and PKC activation proved to be a critical step in the history of PKC since it indicates the implication of PKC in signal transduction between nerve cells and the translation of an extracellular message to an intracellular biochemical signal.

Surveying a variety of tissue sources for PKC, Kikkawa et al. (1982) found that this enzyme had the highest specific activity in brain. PKC was found to be concentrated in brain membranes, whereas it was barely detectable in plasma membrane in peripheral tissue. A greater than 140-fold increase in PKC activity in brain relative to peripheral non-neuronal tissue suggested an important role for PKC in neuronal membranes. But what could this function be?

3.2 Learning and Memory-Related Functions of PKC

The way PKC is stimulated under physiological conditions by Ca^{2+}, phospholipids and DAG and in experimental conditions by phorbol esters implicated PKC in many biological roles in the mid-1980s, ranging from cell growth and differentiation to oncogenesis and inflammation, but there was not yet much attention for PKC in synaptic plasticity and information storage. This started to change after DeRiemer et al. (1985) presented evidence that activation of endogenous PKC by phorbol ester or intracellular injection of the purified enzyme enhanced the voltage-sensitive calcium current in bag cell neurons of the mollusc *Aplysia*. Hence it induced a long lasting enhancement of the excitability of these cells. Before this report, there was no direct evidence for the involvement of PKC in the control of neuronal excitability. Soon thereafter, Baraban et al. (1985) studied the effect of phorbol ester in rat hippocampal slices to further clarify the role of PKC in neuronal function. They found that phorbol ester blocked the late hyperpolarization elicited by synaptic stimulation, and their results also suggested that PKC regulates membrane conductance, possibly through translocation of PKC. These studies clearly implicated PKC in the modulation of membrane function, and it became clear that PKC could be a key regulator of neuronal excitability. It was anticipated in 1986, therefore, that "as with cyclic AMP,

further investigations into the role of PKC in the nervous system will be well rewarded" (Miller, 1986). Furthermore, Routtenberg pointed out in 1985 that "based on the view that plasticity in the adult may be a form of controlled growth regulated by Ca^{2+}, it is attractive to think that such control is mediated, on a brain regional basis, through the PKC system" (Routtenberg, 1985). The above described findings and thoughts were further elaborated in the first studies on models of learning and memory discussed below.

3.2.1 Invertebrate Models for Learning and Memory Clear evidence for a direct link between PKC activation and learning and memory was provided by Farley and Auerbach in January 1986, who used associative learning in the marine snail *Hermissenda* as an invertebrate model for learning and memory. *Hermissenda* can be conditioned to associate a flash of light with rotation that mimics ocean turbulence; in nature, the snail responds to turbulence by flexing its muscular foot to anchor itself to a hard surface. The B photoreceptor cell, which receives the relevant visual and sensory input, contains high levels of PKC. Activation of PKC by phorbol esters or intracellular injection of the purified enzyme in *Hermissenda* B cells induced reduction of K^+ currents similar to these caused by associative learning (Farley and Auerbach, 1986). Some years later, however, concerns were raised about the purity of their used enzyme and their choice of phorbol ester; their enzyme material could have been contaminated with calcium-calmodulin dependent protein kinase and their phorbol ester was not water-soluble, hampering the removal of the agent between measurements (Alkon et al., 1988). The role of PKC in regulation of K^+ channels in *Hermissenda* was further explored in the laboratory of Alkon in the mid and late 1980s. Their results clearly showed that PKC activators mimicked all the electrophysiological changes in the B cell in a manner similar to that observed in the trained animal (Alkon et al., 1986). Microinjection of purified PKC directly into the B photoreceptor cell, concurrent with a flash of light causing a Ca^{2+} load, mimicked the effects of Pavlovian conditioning upon these cells (Alkon et al., 1988). Based on their set of data, they presented a conceptual model of PKC translocation and subsequent regulation of K^+ currents underlying associative learning in *Hermissenda* B cells and speculated about a similar PKC mechanism in vertebrate associative learning.

Besides associative learning in *Hermissenda*, non-associative learning (defensive tail-withdrawal) in the mollusc *Aplysia* has been used as a simple invertebrate form of learning. In 1990, Sacktor and Schwartz showed that short-term sensitization induced translocation of PKC activity from cytosol to membrane in sensory neurons. The attention in the *Aplysia* model was mainly focussed on the facilitation of synaptic transmission (the release of serotonin; Sugita et al., 1992) by PKC during

learning. Compared to associative learning in *Hermissenda*, however, the *Aplysia* model has not been as extensively used to study the role of PKC in learning and memory.

Interestingly, reduced PKC activity measured in brain of the learning mutant fly *turnip* as compared to wild type flies led some researchers to believe that PKC is involved in associative learning in *Drosophila melanogaster* as well (Choi et al., 1991). However, earlier work in the late 1980s studying a different strain of memory-mutant flies (*dunce*) did not reveal such differences in brain PKC activity (Dévay et al., 1989).

3.2.2 Vertebrate Models for Learning and Memory The predominant and most studied vertebrate memory model is long-term potentiation (LTP), first described by Bliss and Lømo in 1973. To date the relationship between LTP and certain aspects of memory has been well documented (Doyère and Laroche, 1992; Bliss and Collingridge, 1993). Hippocampal LTP reflects a persistent enhancement of hippocampal synaptic efficacy, and hence strengthening of neuronal connectivity through synaptic plasticity. In 1985, it was demonstrated in the laboratory of Routtenberg that the phosphorylation of a PKC substrate (F1) was directly related to the plasticity of LTP, and they wrote that "since PKC activity can be regulated for different temporal durations by different mechanisms, it is attractive to think that time-dependent processes of neural plasticity [such as in learning and memory] may be regulated by PKC (Routtenberg et al., 1985; Akers and Routtenberg, 1985). The publication in 1986 that application of phorbol ester to hippocampal slices produced a significant potentiation of synaptic transmission (possibly through enhancement of evoked neurotransmitter release) with many of the characteristics of LTP provided further evidence for a role of PKC in LTP (Malenka et al., 1986; Angenstein & Staak, and Ramakers et al., this issue). Similarly, PKC injections into CA1 hippocampal pyramidal cells elicited features of LTP (Hu et al., 1987). In February 1986, Akers et al. reported that at 1 hour after the induction of LTP, PKC activity was increased twofold in membranes and decreased proportionately in cytosol, suggesting translocation of the activity. They proposed that induction of LTP was accompanied by persistent activation of PKC as this enzyme is known to bind to membranes when activated. These experiments led to the idea that tetanic stimulation of hippocampal synapses activates PKC, which is directly related to the persistence of synaptic plasticity. At that time, they proposed the translocation of PKC activity to be "a novel mechanism regulating the strength of synaptic transmission" (Akers et al., 1986).

In search for a protein that could serve as a molecular substrate of associative memory, these results and those from *Hermissenda* stimulated the analyses of PKC activation in a simple form of associative learning in mammals in Alkon's laboratory in the late 1980s. Rabbits had to learn to associate an

auditory tone with a puff of air to the surface of the eye; the air causes the nictitating membrane to extend, and in time the rabbit extends the membrane when it hears the tone. Bank et al. (1988) found that although total PKC levels were unchanged between trained and control subjects, the percentage of enzymatically measured PKC in the membrane of trained animals was increased. They suggested that there was a long-term translocation of PKC from cytosol to membrane as a result of learning, a mechanism similar to that seen two years earlier by Akers et al. (1986) in LTP. In follow-up experiments in the late 1980s, Olds et al. were able to track PKC changes in rabbit hippocampus employing radioactive phorbol ester (which is believed to specifically bind to membrane-associated PKC; Olds et al., 1989) using a technique developed by Worley and co-workers (1986). Although phorbol esters induce PKC translocation to the membrane, it is thought that "if the concentration of the phorbol ester is kept low enough, the label does not cause translocation of PKC and tags only those neurons with increased amounts of PKC in the cell membrane" (Alkon, 1989). Radioactive phorbol ester binding ([^{3}H]phorbol-12,13-dibutyrate; [^{3}H]PDBu), revealed a redistribution within hippocampal pyramidal cells of the trained animals. They interpreted this finding as a learning-related move of PKC distally along the dendrites, from the cell body (1 day after training) to the basal dendrite (3 days after training). Based on these findings "a multifunctional model was proposed with sequential activation of PKC within different intracellular spatial domains" (Olds and Alkon, 1991), and PKC was supposed to be a nexus in the biochemical events that underlie associative learning.

Several groups in England and Australia used filial imprinting and one trial passive avoidance learning in the one-day old chick as a model system for the molecular and cellular analysis of the substrates of memory. After a preliminary study showing that the application of a PKC inhibitor resulted in the amnesia for the avoidance response (Ali et al., 1988), Burchuladze et al. (1990) published the first report on PKC changes in the intermediate and medial hyperstriatum ventrale (IMHV), the likely brain region for information storage which contains relatively high levels of PKC (Van der Zee et al., 1995). According to the authors, they expected to find a translocation of PKC from the soluble to membrane bound form after passive avoidance in analogue to the situation during LTP. Indeed, a small increase in membrane bound PKC$\alpha\beta$ was found in the left IMHV after training. Thereafter, Sheu et al. (1993) showed a learning-selective increase in the phosphorylation of a membrane-bound PKC substrate in the left IMHV after imprinting, which was possibly related to translocation of PKC to the membrane similarly as seen in LTP.

3.3 Outlasting the Initial Stimulus

Persistent changes in phosphorylation of PKC substrates that outlast an initial signal is most likely an important molecular event in information storage. Prolonged PKC activation, therefore, may serve as a critical step in the chain of biological events leading to memory formation. In the mid-1980s, the group of Routtenberg suggested that the liberation of free cis-fatty acid from membrane phospholipids by phospholipase A_2 (PLA_2) was an important mechanism for PKC activation in LTP. Activation of this pathway was thought to stabilize PKC in an activated state, and thus contributing to the maintenance of the potentiated synaptic response (Linden and Routtenberg, 1989). A synergistic action of free cis-fatty acids and DAG for the activation of PKC was first suggested by Seifert et al., in 1987. In the early part of the 1990s, the group of Nishizuka emphasized that stimuli somewhat separated in time but acting through different types of phospholipases (phosphoinositide- and phosphatidylcholine-specific phospholipase C, and phospholipase D plus a phosphatase; Liscovitch, 1992) and on the same target could induce sustained elevation of DAG (Nishizuka, 1992; Asaoka et al., 1992). PKC activity could be maintained if both DAG and free cis-fatty acid are available at a relatively late phase after the initial signal occurred, which would be essential for long-term cellular responses such as necessary in learning and memory.

In February of 1988, Alkon and Rasmussen published a review with a pivotal role for PKC in a spatial-temporal model of cell activation, which could account for sustained cellular responses as seen in the above described classical conditioning of *Hermissenda* light response and the mammalian eye blink response, and LTP. Persistent enhancement of cell responsiveness after removal of stimuli was suggested to be due to the continued association, or anchoring, of PKC to the membrane. In this way a "cellular memory" is formed, where information has been stored or "remembered" by the cell in the form of constitutively active PKC. In a model of associative learning presented by Bank et al. in 1989, such stable anchoring of PKC to the membrane and hence its prolonged activity was suggested to occur also by phosphorylation of PKC by calcium-calmodulin dependent protein kinase.

In 1988, Bazzi and Nelsestuen (1988a,b) reported that PKC not only translocates upon stimulation, but could also become membrane-inserted and constitutively active. These authors demonstrated *in vitro* that membrane-inserted PKC bound phorbol ester, but that this binding did not further increase the activity of the enzyme. In contrast, the free form of PKC bound phorbol ester only in the presence of Ca^{2+} and phospholipid (subsequently leading to membrane association and membrane insertion). These results indicated that neither Ca^{2+} nor phorbol esters were needed to maintain the conformation of an active PKC complex once the enzyme was inserted into a membrane. Insertion into membranes could be the mechanism by which phorbol ester or DAG induce long-term activation of PKC, which outlasts the

initial activating stimulus for hours or even days. This obviously provided an interesting mechanism for PKC anchoring to the membrane as postulated by Alkon and Rasmussen (Alkon et al., 1988) in their cellular memory model. In a commentary in March 1989, however, Burgoyne pointed out that although it was very attractive that this endogeneously active membrane-inserted PKC could form a cellular memory, it needed to be shown to exist *in vivo*: "Confirmation of the importance of membrane-inserted endogeneously active PKC will require a demonstration that this form of PKC is present in intact cells and that its levels are regulated by signals that result in long term changes in cellular function."

In 1991, receptors for activated protein kinase C (Receptors for Activated C Kinase; RACKs) were identified by Mochly-Rosen and co-workers. Activation of PKC exposes the RACK-binding site. RACKs can bind PKC at different subcellular sites, among which the plasma membrane. Although the concept of intracellular receptors for activated PKC seems appealing in terms of the induction of a more permanent localization of PKC to the membrane (and hence providing an additional mechanism to outlast the initial activating stimulus), to our knowledge no studies have been published linking RACKs to learning and memory processes.

4. A Brief Outline of Ongoing Research on PKC in Learning and Memory in the 1990s

After the initial studies in the mid-1980s a vast number of experiments have further substantiated the role of PKC in learning and memory and LTP using molecular genetic, behavioral, pharmacological, electrophysiological or immunocytochemical approaches. Unraveling the role of PKC in learning and memory may eventually illustrate how synaptic plasticity and information storage might be reflected in the molecular properties of PKC. A brief outline of experiments performed in the 1990s is presented below describing some of the highlights of this research.

4.1 Continuing Studies on Synaptic Plasticity and PKC Redistribution

In the early 1990s, part of the research on the role of PKC in LTP focussed on whether the locus of PKC activity was with the pre- or postsynaptic components during different phases of LTP. A differential pre- and postsynaptic localization of PKC isoforms was suggested to underlie the selective sequential activation observed in LTP (Huang et al., 1992), stating that "it is attractive to think that these two events [LTP induction and persistence] are sequentially activated and employ different PKC

subtypes differentially localized to presynaptic and postsynaptic elements". Indeed, earlier immunocytochemical studies using PKC isoform-specific antibodies had revealed a somewhat differential pre- and postsynaptic localization of PKC subtypes in rat hippocampus (Saito et al., 1989; Ito et al., 1990; Kose et al., 1990). Interestingly, a sharp differential distribution of PKCγ versus PKCαβ was found in respectively a post- and presynaptic localization in the IMHV of the chick brain mentioned above, where LTP-like phenomena could play a role in the information storage function of this region (Van der Zee et al., 1995).

Several mechanisms inducing persistently active forms of PKC other than through translocation of PKC from cytosol to membrane were found in LTP. Proteolytic activation of PKC, resulting in a constitutively active fragment called PKM (Suzuki et al. 1992; Sactor et al. 1993), oxidative modification of PKC (Palumbo et al., 1992) and the phosphorylative activation of PKC (Klann et al., 1993) were described as mechanisms explaining the long-term activation of PKC in different phases of LTP. In a commentary in September 1993, in response to this research, Schwartz referred to PKC as a "cognitive kinase", and wrote that "in a metaphorical sense, PKC behave as if they were taught, memorizing temporal connections between events in the empirical world" (Schwartz, 1993).

In 1994, Angenstein et al. were the first to report a translocation *from the membrane to the cytosol* in LTP, demonstrating that the translocation cascade may be more complex than previously thought. In this respect it is worth noting that the suggestion that prolonged translocation of PKC from the soluble to an integral membrane protein form plays an important role in memory processes (as described in section 3.3) has recently been questioned. The doubt is primarily based on the observation of rapid redistribution of phorbol ester-induced membrane-associated PKC back to the cytosol after the dissociation of the applied phorbol esters *in vivo* (Szallasi et al., 1994; Mosior and Newton, 1995).

Continuing studies in the early and mid-1990s in Alkon's laboratory using [^{3}H]PDBu to track learning-specific alterations in PKC showed with increasing complexity that the type and time-point of (spatial) learning in rats determined the degree and direction of the changes in [^{3}H]PDBu binding (Olds et al., 1990; Golski et al., 1995). The [^{3}H]PDBu technique was also used as a marker for PKC in the B photoreceptor cells of *Hermissenda* associated with Pavlovian conditioning, and it was shown that conditioning increased [^{3}H]PDBu-binding in molluscan cells in a fashion comparable to mammalian neurons (McPhie et al., 1993).

4.2 PKC Activity Measures

PKC activity measures have provided a good parameter to study the contribution of PKC in a number of different approaches. Laboratories in Italy studied the role of PKC in aging-related memory deficiences. One of the first reports on age-related changes in PKC activity, translocation and physiological response to PKC stimulation appeared in the late 1980s (Friedman and Wang, 1989). Thereafter, Battaini and co-workers analysed in detail age-related alteration in PKC functioning in brain (Battaini et al., 1994). A behavioral-genetic analysis was first used in the early 1990s by the group of Wehner. Inbred strains differing in spatial memory performance were shown to have different levels of hippocampal PKC activity (Wehner et al., 1990), and Fordyce and Wehner (1993) showed that age-related deficits in spatial learning performance correlated with a decline in hippocampal PKC activity using two inbred strains. These results suggest a genetic correlation between PKC activity and learning abilities. The use of different inbred strains of mice proved to be a valuable approach in combination with aging and pharmacology to study the role of PKC in learning and memory. Detailed PKC activity measures were also used in the mid-1990s in the group of Jaffard in France searching for a correlation between hippocampal PKC activity and the learning abilities of mice (Noguès et al., 1994; 1996b). Causal models are proposed by the authors to explain their results and the seemingly contradictory data on the role of PKC in learning and memory in the literature (for a new model, see Noguès, this issue).

4.3. Pharmacological Manipulations

Besides the various experiments using different PKC inhibitors or activators (phorbol esters) to study electrophysiological and biochemical alterations at the cellular level, a pharmacological approach has been used to directly interfere with memory performance in animals. As phrased by Noguès et al., (1996a) "the rationale is to block or to activate PKC activity in an attempt to inhibit or to facilitate memory processes". One of the first studies employing PKC inhibitors was performed by Ali et al. (1988) using one trial passive avoidance training in the chick. Application of the inhibitors to brain resulted in the amnesia for the avoidance response. One of the first examples of the effect of intraventricular application of PKC inhibitors to rodents is provided by Takashima et al. (1991), showing that it caused a marked impairment in one-trial passive avoidance response and spatial learning in rats. It should be taken into account, however, that some of the inhibitors used in the early studies often displayed activity towards other kinases than PKC, and nonspecific effects on the kinase or the cell membrane could be exerted by some of them due to their hydrophobic nature. Moreover, due to the wide functional cellular role of

PKC, the effects of inhibition of PKC activity should not a priori be taken as evidence that the enzyme plays a role in learning and memory. Using the approach of PKC activation employing phorbol esters, injectecting it either into the lateral ventricles prior to training (Paylor et al., 1991) or directly into the hippocampus immediately after training (Yang and Lee, 1993) improved the learning performance of rats. Taken the pharmacological data over the years together, both in rodent (Noguès et al., 1996a) and chick brain (Zhao et al., 1994) the results indicate that PKC activity is related to information storage.

4.4 Some New Approaches

An immunocytochemical approach was used by us in Luiten's laboratory to study the contribution of Ca^{2+}-dependent PKC isoforms in learning and memory. In 1992, the authors demonstrated that spatial learning in mice and rats induced PKCγ-specific alterations in the hippocampus, whereas no such changes were found for PKCαβ using an antibody that did not discriminate between PKCα, β1 and β2 (Beldhuis et al., 1992; Van der Zee et al., 1992). Different types of learning tasks stimulating different (but partly overlapping) brain circuits induced corresponding patterns of altered PKCγ-immunoreactivity (Luiten et al., 1991; Van der Zee et al., 1992; 1994); these *in situ* alterations in immunoreactivity enabled us to map the neural substrate and the individual neurons engaged in the learning task. Thereafter, a battery of Ca^{2+}-dependent PKC isoform-specific antibodies were used in Disterhoft's laboratory to study immunocytochemical changes in the rabbit hippocampus after associative learning. Like in spatial learning in rodents, the results pointed to PKCγ as the main Ca^{2+}-dependent isoform involved in learning and memory processes. The combined approach of behavior, immunocytochemistry and biochemistry led to a proposed model for the role and molecular cascade of PKCγ in associative learning (see Van der Zee et al., this issue).

In the early 1990s, PKCγ mutant mice became available and were tested for hippocampal LTP and spatial and contextual learning. Although LTP was greatly diminished in these mice, it appeared that PKCγ is "not part of the molecular machinery that produces LTP but is a key regulatory component" (Abeliovich et al., 1993a). The behavioral results showed only relatively mild deficits (Abeliovich et al., 1993b). These results seem to suggest that although PKCγ is important for normal hippocampal function in learning and memory, it can be compensated for when its expression is shut off before the hippocampus is functionally developed. However, our knowledge of developmental functions of PKC is

rather limited and more extensive investigations are needed (for some details on the role of PKC in development of the optic tectum/superior colliculus, see McCrossan et al., this issue).

5. Conclusion

Ever since the discovery of PKC extensive biochemical analyses has revealed a complex cascade of events that underlie enhanced and sustained activation of PKC (Nishizuka 1995), which is thought necessary to explain the putative role of this enzyme in persistent cellular changes associated with learning and memory processes. The search for the role of PKC in learning and memory, and especially a persistently active or autonomous form that makes the enzyme less dependent on second messengers and thereby prolonging its action well beyond the initial stimulating event, will continue. The ultimate goal of PKC activation, protein phosphorylation in a correct spatial-temporal fashion, has focused attention to the target substances. Since the discovery in the mid-1970s of a major PKC substrate participating in synaptic plasticity (F1, also referred to as GAP43 or B50; Routtenberg and Ehrlich, 1975; Zwiers et al., 1976) an impressive differentiation of over 100 enzyme targets and numerous physiological actions have been described (Liu, 1996; Casabona and Ramakers et al., this issue).

Compared to the immense number of PKC-related publications (over 17,000), the total amount of papers on PKC and learning and memory, starting to appear in the mid-1980s, only consists of about 140 (Fig. 1), making up less than 1 % of all PKC-related reports. In turn, only approximately 3 out of every 1000 papers on learning and memory are PKC-related. Notwithstanding these modest numbers, the past decade or so of PKC research and the reviews in this special issue clearly show the exciting aspects of the putative role for PKC in synaptic plasticity and information storage and its impact on learning and memory research.

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